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Mounting a specific immune response increases energy expenditure of the subterranean rodent *Ctenomys talarum* (tuco-tuco): implications for intraspecific and interspecific variation in immunological traits

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SUMMARY

It was recently hypothesised that specific induced defences, which require substantial time and resources and are mostly beneficial against repeated infections, are more likely to be favoured in 'slow-living-pace' species. Therefore, understanding how different types of immune defences might vary with life history requires knowledge of the costs and benefits of defence components. Studies that have explored the energetic costs of immunity in vertebrates have done so with a focus primarily on birds and less so on mammals, particularly surface-dwelling rodents. In this study, we evaluated whether an experimental induction of the immune system with a non-pathogenic antigen elevates the energetic expenditure of a subterranean rodent: *Ctenomys talarum* (tuco-tucos). In both seasons studied, a significant increase in oxygen consumption was verified in immune-challenged tuco-tucos injected with sheep red blood cells (SRBC) compared with control animals. The increase in oxygen consumption 10 days after the exposure to SRBC was lower for female tuco-tucos monitored in the breeding season compared with females in the non-breeding season. Interestingly, antibody titres of female tuco-tucos did not decrease during the breeding season. Our results add new insight into the role of other factors such as basal metabolic rate or degree of parasite exposure besides 'pace of life' in modulating the interspecific immunological variation observed in natural populations of mammals.

Key words: antibody response, metabolic costs, life history.

INTRODUCTION

Immune defence is not only expected to represent fitness benefits in the form of resistance against pathogen infections but also substantial costs (Lochmiller and Deerenberg, 2000). In this context, variability in immune strategies is expected to arise from the subsequent trade-offs between allocating resources to immunity *vs* other costly processes (Sheldon and Verhulst, 1996; Lee et al., 2005; Martin et al., 2008). However, the assumption of a fitness cost to resistance has been questioned (Rigby et al., 2002), triggering a debate over the occurrence and importance of immunological costs in animals and the role of environmental and life-history traits in modulating the costs of resistance (Sandland and Minchella, 2003).

Understanding how different types of immune defences might vary with life history therefore requires knowledge of the costs and benefits of defence components. The vertebrate immune system comprises the innate and the adaptive branches. The adaptive immune system represents the second line of defence and is generally divided into a cell-mediated component – which fights primarily against intracellular pathogens such as viruses (Janeway et al., 2004) – and a humoral component – which recognises and destroys extracellular parasites and pathogens through the concerted action of B- and Th2-cells that differentiate and produce antibodies (Janeway et al., 2004). While induced antibody and cell-mediated immune responses are considered developmentally expensive, a major benefit of humoral immunity is the storage of pathogen recognition information in the form of immunological memory (Janeway et al., 2004).

Based on the relative costs of the two immune defence arms, Klasing (Klasing, 2004) and Lee (Lee, 2006) proposed that specific induced defences, which require substantial time and resources and are mostly beneficial against repeated infections, are more likely to be favoured in 'slow-living-pace' species – with high per-offspring investment, more extensive developmental times and longer lifespans – rather than in 'fast-living-pace' species (see also Martin et al., 2006a; Martin et al., 2006b). Factors other than life-history traits contribute to shape immune defences (Lee, 2006). Between species, two main factors have been proposed to explain the reliance on a particular defence strategy: pathogen exposure (Lindström et al., 2004; Lee, 2006) and body size (Lee, 2006). Particularly, species with high parasite prevalence [estimated by the number of hosts infected divided by the number of hosts examined (see Bush et al., 1997)], and hence more frequent exposure to parasite infections, should show greater investment on adaptive antibody responses, which represent an advantage in the face of future infections, rather than on non-specific induced defences (Shudo and Iwasa, 2001). However, large-bodied species with lower mass-specific metabolic rates should also be able to invest more on developmentally costly induced immune responses in comparison with smaller-bodied species with high mass-specific metabolic rates (Lee, 2006). Taken together with life-history data, these additional factors should provide a more detailed understanding of immune system variation than life-history traits alone.

Among vertebrates, the effect of mounting an immune response on energy expenditure has been explored with a focus primarily on birds and less so on mammals, particularly surface-dwelling rodents (Demas et al., 1997; Svensson et al., 1998; Ots et al., 2001; Cichón et al., 2002; Raberg et al., 2002; Bonneaud et al., 2003; Derting and Compton, 2003; Martin et al., 2004; Derting and Virk, 2005; Eraud

et al., 2005; Pilorz et al., 2005; Amat et al., 2007). As Lee (Lee, 2006) has pointed out, further work is needed to include additional study cases, especially non-model species, into the working framework of the physiological implications of immune defence.

The South American subterranean rodents *Ctenomys talarum* (Talas tuco-tucos, Thomas 1898) live in permanently sealed burrows and most of their activities are restricted to these tunnels (Busch et al., 2000). Tuco-tucos maintain exclusive territories and are mostly sedentary (Busch et al., 1989). The relatively low parasite richness [defined as the number of parasite species recorded in a host population (Bush et al., 1990)] of gastrointestinal helminths observed in this species (Rossin and Malizia, 2002) and other subterranean rodents has been related to their solitary existence (Nevo, 1999). However, the high prevalence of parasitic infections, and therefore high pathogen exposure, in *C. talarum* led other authors to consider the possibility that burrow systems may provide physical conditions (i.e. moisture, low ventilation and protection from UV light) that favour high levels of parasite transmission (Rossin and Malizia, 2002). Additionally, the moist and stagnant conditions of the subterranean environment are hypothesised to have favoured low basal metabolic rate (BMR), observed in *C. talarum* (Busch, 1989; Luna et al., 2002) and other subterranean rodents, as an adaptation to avoid overheating during digging (McNab, 1966) or to cope with the costs of burrowing (Vleck, 1979; Luna and Antinuchi, 2007; Luna et al., 2009). This low BMR has been associated to other singular traits of this species: tuco-tucos live 'slow pace' reproductive lives; females have a long gestation period (95 days) and give birth to altricial pups (Zenuto et al., 2002a) only twice a year (Busch et al., 1989).

The goal of this study is to assess whether an experimental induction of the immune system with a non-pathogenic novel antigen elevates the energetic expenditure of the subterranean rodent *C. talarum*. Considering the characteristics of the energetic metabolism and life history of this species, we hypothesise that tuco-tucos rely strongly on specific humoral immune responses and less so on constitutive immune defences. Under this hypothesis, we expect (a) that tuco-tucos mount a strong adaptive humoral response when presented to a novel antigen, (b) that the ability to mount a specific immune response is negatively affected in females during the reproductive season, considering the high per-offspring investment of tuco-tucos, and (c) that the energetic investment allocated to mounting such a humoral response is greater in tuco-tucos than in surface-dwelling rodents which, in comparison, live 'fast pace' reproductive lives and have higher metabolic rates. This study represents the first attempt to assess the energetic costs associated with immunity in a subterranean rodent.

MATERIALS AND METHODS

Animal capture and captivity conditions

Adult *C. talarum* (tuco-tucos) were caught with plastic tube traps during the non-reproductive (mid February to mid April 2008) and the reproductive seasons (September to early December 2008) in the locality of Mar de Cobo, Buenos Aires Province, Argentina (37°46′S 57°27′W). Only pregnant females were brought to the lab during the reproductive season; nursing females were immediately returned to their burrows after capture to avoid distressing the pups left in the nest. Animals were transported to the Laboratory of Ecophysiology at the National University of Mar del Plata where they were weighed and put in individual plastic boxes with wood shavings as bedding. Animals were fed a mixed diet of grass, alfalfa, lettuce, corn, sweet potatoes, carrots and sunflower seeds *ad libitum*. Animal room conditions (temperature and photoperiod) were controlled automatically. Tuco-tucos remained captive for the duration of the experimental assays (*ca*. 4 weeks) after which they were released at the point of capture. All field and laboratory procedures conformed to institutional (National Council for Scientific and Technological Research, CONICET, and National Agency for Scientific Promotion: PICT 1992) and American Society of Mammalogists guidelines (Gannon et al., 2007) for the capture, handling and use of mammals.

Immunisation

Immediately after capture, animals were randomly assigned to two groups: control (C) and immune challenged (IC). Animals remained in captivity for 10 days before receiving the first injection because a previous study showed that this is the necessary time lapse for animals to acclimatise to captivity conditions and, hence, lower their stress levels (Vera et al., 2008). The capture and transport to the laboratory constitutes a very strong stressor for tuco-tucos, causing a marked increase in the neutrophyl:lymphocyte ratio (N:L). After 10 days in captivity, N:L ratios decrease to values similar to those monitored in the field (Vera et al., 2008), and cortisol levels remain similar to field values (F. Vera, R.R.Z. and C.D.A., unpublished). Therefore, 10 days after capture (day –1), oxygen consumption ($\dot{V}_{O_2}$) of animals from both groups was measured as described below. The following day (day 0), animals of group C were weighed and injected intra-peritoneally with sterile phosphate buffered saline solution (PBS, $1.5\,\mu l\,g^{-1}$ of animal mass), and animals of the IC group were weighed and injected intra-peritoneally with sheep red blood cells (SRBC – Sigma R3378, Sigma Chemical Co., St Louis, MO, USA, 10% suspension, $1.5\,\mu l\,g^{-1}$ of animal mass). SRBC is a non-pathogenic antigen known to trigger T- and B-lymphocyte-dependent immune responses (Bacon, 1992). The strength of the immune response against SRBC is considered indicative of resistance to extracellular infections [e.g. bacteria, macroparasites, (see Deerenberg et al., 1997)]. After the injection, we collected blood (~200 µl) from the retro-orbital sinus for hemoagglutination assays performed as described below.

Seven days post-injection (d.p.i.), animals were weighed again and we collected another blood sample. Preliminary trials conducted on nine adult tuco-tucos injected with SRBC, whose immune response was monitored at 3, 7, 10 and 14 d.p.i. using hemoagglutination assays, showed a peak in antibody titres 7 d.p.i. (A.P.C. and R.R.Z., unpublished). These trials showed that *C. talarum* exhibit low levels of primary immune response to SRBC. Therefore, we used two injections of SRBC in this study to sufficiently stimulate the immune system and, consequently, enhance the potential for detection of and increase in energy expenditure associated with immune defence (see Derting and Virk, 2005). To do this, at day 7 tuco-tucos from both experimental groups were injected again with PBS (C group) or SRBC (IC group), following the protocol described above. Because two injections were used, energy expenditure on mounting the immune response after the second injection (see below) included some costs of a secondary immune response as well as the primary immune response. Fourteen days after the first injection, animals were weighed and we collected blood for the last time.

Hemoagglutination assays

Antibody production was assessed by a hemoagglutination assay in 96-well microplates (Corning Star Catalog No. 3798, Corning, NY, USA). Immediately after collection, blood was kept at 4°C until it was centrifuged at 3000 r.p.m. for 15 min, after which plasma was separated and heated at 56°C for 30 min to inactivate the complement. Plasma was stored at –20°C until used in the hemoagglutination assay. 20 µl of heat-inactivated plasma extracted

at days 0, 7 and 14 was added to 20 µl of PBS in the first well of the plate; serial dilutions in PBS (1:2–1:256) were then carried out, followed by the addition of 20 µl of a 1% suspension of SRBC to each well. The plates were gently agitated for 1 min and then incubated at 37°C for one hour. After that, plates were kept still at 4°C for 2 h before macroscopic examination for agglutination was performed. For comparative purposes, antibody titres were expressed as the negative $\log_2$ ($-\log_2$) of the minimum plasma concentration that contained enough antibody to agglutinate the antigenic SRBC.

Measurement of $\dot{V}_{O_2}$

$\dot{V}_{O_2}$ measurements were performed a day before the first injection (day –1) and 4 and 10 days after the first injection. We scheduled $\dot{V}_{O_2}$ measurements on these days in order to estimate the cost of mounting an immune response against SRBC in tuco-tucos while this was developing, before the peak response was achieved 7 and 14 d.p.i.

$\dot{V}_{O_2}$ of animals was measured in 90 min trials using a computerised positive-pressure open-flow respirometry system (Sable System, Las Vegas, NE, USA) on days –1, 4 and 10. $\dot{V}_{O_2}$ was measured at 25°C [within the range of thermoneutrality for *C. talarum* (Busch, 1989)] in the morning or in the afternoon, because *C. talarum* exhibits an arrhythmic pattern of daily activity (Luna et al., 2000). Animal body temperature was recorded after measuring $\dot{V}_{O_2}$ (Cole Parmer Thermistor 8402-10, YSI probe model 93k73545-402, Vernon Hills, IL, USA; nearest 0.1°C).

The metabolic system consisted of a cube-shaped chamber with double aluminium walls, which contained polyurethane in between them. Temperature was adjusted automatically by a computerised system (nearest 1°C) using two Peltier intercoolers (Melcor, model CP-1.4-127-061, Cleveland, OH, USA). The chamber received dry and CO_2-free air at 1500 ml min^{-1} from a mass flow controller (Sierra Instruments, Monterey, CA, USA) to ensure chamber equilibrium (Lasiewski et al., 1966). Air passed through a CO_2 absorbent (IQB, Quimica Kubo, Mar del Plata, BA, Argentina) and a water scrubber (Silica Gel, Quimica Kubo) before and after passing through the chamber. Excurrent air from the metabolic chamber was sub-sampled at 180±10 ml min^{-1}, and $\dot{V}_{O_2}$ was obtained from an Oxygen Analyzer FC-1B every second set by a Datacan V-PC program (Sable System). The baseline of the respirometry system was set in 20.95% of oxygen before the beginning of each experiment. Rate of oxygen was calculated using eqn 4a from Withers (Withers, 1977). Resting metabolic rate (RMR) was measured as the 5-min lowest steady-state values of the last 30 min of a 90-min trial (Luna and Antinuchi, 2007). For comparative purposes, data were expressed as mass-specific RMR (ml O_2 g^{-1} h^{-1}) and kilojoules (KJ). An equivalent of 20.08 J ml^{-1} O_2 was used to convert $\dot{V}_{O_2}$ to energy values (Schmidt-Nielsen, 1990).

Determination of leukocyte profile

Analyses of leukocyte diversity and abundance were used to assess innate immune defences in *C. talarum*. Because leukocytes are directly involved in the mammalian immune response to pathogens (Roitt et al., 1998), estimates of levels of circulating leukocytes provide a global estimation of immune activity. Further, because different types of leukocytes perform different functions, the relative abundance of leukocyte types can be used to draw inferences about the roles of acute *vs* chronic immune challenges in shaping the immune response (Nunn et al., 2000). Additionally, N:L is a known chronic stress indicator (Davis et al., 2008), which could serve to monitor the animals' stress status while in captivity (see Vera et al., 2008). Leukocyte abundance and diversity was quantified following standard protocols (Voigt, 2000). Briefly, blood smears were obtained from all animals (a) in the field, less than 15 min after they were trapped by obtaining a blood drop from the tip of the tail, and (b) in captivity, at days 0, 7 and 14 from the blood sample collected. Blood smears were fixed in 70% methanol for 10 min, then stained with May-Grumwald Giemsa solution and examined under oil immersion at ×100 magnification (Olympus CX 31, Tokyo, Japan). The cells were counted only across the entire monolayer area of the slide ['wandering technique' (Voigt, 2000)]. All cell types were identified according to the characteristics of their morphology described in standard laboratory mammals (Voigt, 2000) and previous data for the species (Vera et al., 2008): lymphocyte, neutrophil, eosinophil, basophil and monocyte. Leukocyte counts were obtained by recording the number and kind of each cell type until a cumulative number of 200 cells was reached; then, the relative count for each cell type over the total number of leukocytes and the N:L ratio were calculated. Also, in a single pass along the slide, the number of erythrocytes and leukocytes encountered in 30 fields was recorded and the number of total leukocytes was standardised to 100,000 erythrocytes (Bachman, 2003). Leukocyte count determinations were performed by one person (R.R.Z.). All smears were inspected once, and then a random sample, which represented half of the number of smears inspected, was selected to repeat the procedure.

Haematocrit

Haematocrit is the proportion of blood volume occupied by packed red blood cells and is considered to reflect the animal's condition; specifically, haematocrit is thought to be affected by ecological conditions and exercise as well as blood parasites (e.g. Pasquinelli, 1971). We measured haematocrit of the animals on days 0, 7 and 14, following standard protocols (Voigt, 2000). Briefly, blood was collected from the retro-orbital sinus in a heparinised capillary tube, which was then centrifuged at 14,000 r.p.m. for 15 min (Cavour VT 1224 centrifuge, Buenos Aires, Argentina). The haematocrit was assessed as the proportion of capillary length occupied by packed red blood cells in relation to capillary length occupied by all blood components (Abaco CAV 1224, Ciudad de Buenos Aires, Argentina). Haematocrit was determined in duplicates and the resulting values were averaged.

Statistics

All tests were performed in Statistica (Statsoft, Tulsa, OK, USA) using $\alpha>0.05$ to reject the null hypothesis. Throughout the text, results are expressed as means ± standard deviation (±s.d.). A two-way repeated-measures analysis of variance (RM-ANOVA) was used to evaluate the effect of season (reproductive *vs* non-reproductive) and sex on antibody titres among days of treatment.

A Student's *t*-test was used to assess if body mass differed significantly between the experimental groups at the beginning of the experimental trials. A three-way repeated-measures analysis of covariance (RM-ANCOVA) was used to evaluate the hypothesis of no differences in RMR of tuco-tucos (ml O_2 h^{-1}) between experimental groups (C *vs* IC), sexes (males *vs* females) and season (non-reproductive *vs* reproductive) and among different days of treatment (–1, 4, 10), using body mass (g) as the covariate.

More specifically, to evaluate the effect of season (non-reproductive *vs* reproductive) and sex on the percentage increase of RMR in IC animals, a two-way RM-ANOVA was used. Finally, a one-way RM-ANOVA was used to evaluate the hypothesis of no differences in body mass change between experimental groups (C *vs* IC).

The Structural Equation Modelling (SEPATH) module of Statistica (Statsoft) was used to conduct a path analysis, which is an extension of a multiple linear regression analysis that allows the decomposition and interpretation of linear relationships among measured variables and to test the overall path diagram as a likely cause of observed data (Legendre and Legendre, 1998). Path analysis has been used in studies performed in natural conditions to evaluate the relationship between environmental variables and reproduction (e.g. Fanjul et al., 2006) as well as, more recently, within an experimental context (Nespolo et al., 2003). We used this analysis to model the individual RMR (ml $O_2\,h^{-1}$), intensity of the immune response (measured as $-\log_2$ antibody titre) and partial correlation with body mass (g). In particular, two models were tested: (1) 'direct' model, which consisted of this causal structure (body mass → RMR, intensity of immune response → RMR), and (2) 'indirect' model, which consisted of this causal structure (body mass → RMR, body mass intensity of immune response → RMR). The second model considered an additional path from 'body mass' to 'antibody titre'. In this way, the effect of 'body size' over RMR was decomposed into direct effects, as in the first model, as well as indirect effects *via* 'antibody titre'. Both models were tested separately in the reproductive and the non-reproductive seasons, for measurements corresponding to day 10 (RMR) and day 14 (intensity of immune response) after first exposure to the antigen. We used maximum likelihood to compute path coefficients (β), with their respective standard errors. A Student's *t*-test was used to examine if path coefficients were significantly different from zero. To test the fit of the overall path diagram, we used a χ^2 goodness-of-fit test, rejecting the models with $P<0.05$, as well as the Akaike Information Criterion (AIC).

A two-way RM-ANOVA was used to evaluate the hypothesis of no differences in (a) total leukocyte counts, (b) relative lymphocyte counts, (c) relative eosinophil counts, and (d) $1/x^{0.5}$-transformed N:L ratio between sexes, seasons (non-reproductive *vs* reproductive) and between the field and day 0 of treatment. In order to evaluate if the strength of the immune response was positively correlated with the animal's stress level (estimated by N:L ratios) at days 7 and 14, the effects of body mass on antibody titre and N:L ratios were first removed by using the residuals of a linear regression of antibody titre and N:L ratios on body mass. Values of body mass and antibody titres were log-transformed to achieve linearity. Residual antibody titres were correlated with residual N:L ratios.

A three-way RM-ANOVA was used to evaluate the hypothesis of no differences in mean haematocrit values of tuco-tucos between experimental groups (C *vs* IC), sexes, seasons (non-reproductive *vs* reproductive) and among different days of treatment (0, 7, 14).

RESULTS

A total of 56 adult *C. talarum* were caught. Of those, 46 (10 males and 10 females in the non-reproductive season; 13 males and 13 females in the reproductive season) were assigned randomly to either the IC or the C group. The remaining 10 animals could not be included in the study because (a) three died during the experiment, (b) six lost a significant amount of weight during the first 10 days of captivity, and (c) one was accidentally injected with the wrong volume of PBS. Animals from b and c were released at their point of capture once they had recovered to their initial weight at the moment of capture. We first evaluated if body mass at the beginning of the experiment differed between the experimental groups. A Student's *t*-test confirmed that tuco-tucos were assigned randomly to both experimental groups, at least with respect to body mass (non-reproductive season: $t=-1.64$, d.f.=18, $P=0.118$; reproductive season: $t=-1.88$, d.f.=24, $P=0.072$).

Immune response

In both seasons, none of the animals from the C group mounted an immune response against SRBC at days 0, 7 or 14, as expected if they had never been exposed to this antigen before. In the IC group, animals did not mount an immune response at day 0. Antibody titres of IC animals were significantly different between 7 and 14 d.p.i. (two-way RM-ANOVA: d.f.=1, $F=66.43$, $P<0.0001$, Fig. 1). Significant interactions were detected between sex and d.p.i. (two-way RM-ANOVA: d.f.=1, $F=4.55$, $P=0.039$, Fig. 1) and between sex and season (two-way RM-ANOVA: d.f.=1, $F=4.92$, $P=0.032$, Fig. 1), although no significant effects were found when the Scheffé *post-hoc* tests were performed for these situations. No significant interaction was verified among sex, season and days after injection (two-way RM-ANOVA: d.f.=1, $F=0.00$, $P=0.968$, Fig. 1). No significant differences in antibody titres were observed between the reproductive and the non-reproductive seasons (two-way RM-ANOVA: d.f.=1, $F=0.76$, $P=0.388$).

Metabolic costs of mounting an immune response

RMR differed significantly between experimental groups, between seasons and among days after the first injection. However, sex did not have a significant effect (Table 1). The interactions 'd.p.i. × season' and 'd.p.i. × treatment' had a significant effect on RMR (Table 1). As expected, at day –1, before the injection, RMR did not differ between experimental groups (Scheffé, $P>0.05$). Four d.p.i., the RMR of IC tuco-tucos was significantly higher than that at day –1 (159.14±37.83 *vs* 128.37±31.16 ml $O_2\,h^{-1}$, respectively, Scheffé $P<0.001$). Ten d.p.i., the RMR of IC animals was significantly higher compared with that at day –1 (160.22±33.14 *vs* 128.37±31.16 ml $O_2\,h^{-1}$, respectively, Scheffé $P<0.001$) but did not differ significantly from that measured at 4 d.p.i. (Scheffé $P>0.05$). In control animals, there were no significant differences in RMR among days (Scheffé, $P>0.05$ for all comparisons). Finally, RMR was significantly higher in the reproductive season, compared with the non-reproductive season (150.45±34.9 *vs* 140.56±28.3 ml $O_2\,h^{-1}$, Table 1).

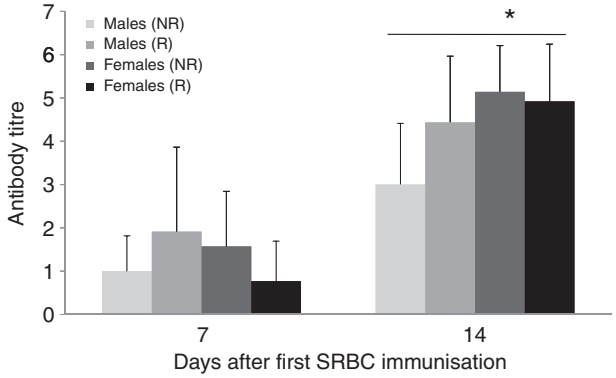

Fig. 1. Titres of antibodies (means + s.d.) against sheep red blood cells (SRBCs) in male and female tuco-tucos at days 7 and 14 after the first injection during the reproductive (R) and the non-reproductive (NR) seasons. Antibody titres are expressed as $-\log_2$ of the minimum plasma concentration that contained enough antibody to agglutinate the antigen. Asterisk denotes significant differences between days of treatment (RM-ANOVA, $P<0.05$).

Table 1. Results of three-way repeated-measures analysis of covariance (ANCOVA) performed to evaluate the hypothesis of no differences in RMR of tuco-tucos (ml O_2 h^{-1}) between experimental groups, sexes and seasons and among different days of treatment (–1, 4, 10), using body mass (g) as the covariate

	d.f.	*F*	*P*
Between effects			
Treatment (SRBC *vs* control)	1	9.660	0.004*
Sex (males *vs* females)	1	1.370	0.250
Season (reproductive *vs* non-reproductive)	1	5.720	0.022*
Sex × season	1	0.004	0.944
Sex × treatment	1	0.001	0.977
Season × treatment	1	1.980	0.168
Sex × season × treatment	1	0.002	0.965
Within effects			
d.p.i.	2	3.940	0.024*
d.p.i. × sex	2	0.917	0.405
d.p.i. × season	2	2.992	**0.057**
d.p.i. × treatment	2	4.476	0.015*
d.p.i. × sex × season	2	2.837	**0.066**
d.p.i. × sex × treatment	2	1.694	0.191
d.p.i. × season × treatment	2	0.561	0.573
d.p.i. × sex × season × treatment	2	1.318	0.274

Asterisks denote significant effects. Marginally significant effects are presented in bold. SRBC: sheep red blood cells. d.p.i., days post-injection.

Table 2. Path models adjusted to measurements of resting metabolic rate (RMR), intensity of immune response and body mass, obtained from tuco-tucos injected with sheep red blood cells during the reproductive (R) and the non-reproductive (NR) seasons

Season	Model	χ^2	d.f.	*P* value	AIC
NR	Direct	9.67	3	**0.02**	1.74
	Indirect	5.23	2	0.07	1.47
R	Direct	1.54	3	0.67	0.63
	Indirect	0.59	2	0.74	0.72

AIC: Akaike Information Criterion. Significant *P* values are denoted in bold.

The mean percentage increase in RMR of tuco-tucos injected with SRBCs did not differ between the reproductive and non-reproductive seasons (two-way RM-ANOVA, d.f.=1, F=2.23, P=0.153) or between the sexes (two-way RM-ANOVA, d.f.=1, F=1.04, P=0.322) but there was a significant interaction between season, sex and days after injection (two-way RM-ANOVA, d.f.=1, F=8.96, P=0.008). The mean percentage increase in RMR 10 days after the first immunisation was higher for females in the non-reproductive season compared with those monitored in the reproductive season (Scheffé, P=0.04).

In the non-reproductive season, the better-adjusted model was that represented by the path diagram that included direct and indirect effects (*via* 'intensity of immune response') of body mass over RMR (Table 2). This 'indirect' model was more explanatory than the 'direct' model and this was further supported by the fact that all paths from body mass were significant (Fig. 2A). On the contrary, in the reproductive season, the direct and indirect model provided a similar fit (Table 2), with a slightly higher AIC for the 'direct' model (Fig. 2B).

Leukocyte profile

After 10 days in captivity, tuco-tucos showed a decrease in their total leukocyte counts compared with values obtained in the field; animals in the non-reproductive season showed lower counts than those in the reproductive season but there were no differences between sexes (two-way RM-ANOVA: $d.f._{sex}$=1, F=1.26, P=0.268; $d.f._{season}$=1, F=7.91, P=0.007; $d.f._{lab\ vs\ field}$=1, F=30.28, P<0.001, Fig. 3). This decrease in captive animals was also verified in relative eosinophil counts, suggesting a negative effect of captivity on these parameters, but sex and season did not have a significant effect (two-way RM-ANOVA: $d.f._{sex}$=1, F=2.08, P=0.157; $d.f._{season}$=1, F=0.79, P=0.378; $d.f._{lab\ vs\ field}$=1, F=29.56, P<0.001, Fig. 3). By contrast, the N:L ratio was significantly lower in tuco-tucos in captivity compared with those in the field, as expected if captivity does not represent a significant stressor factor; sex and season did not have a significant effect (two-way RM-ANOVA: $d.f._{sex}$=1, F=3.13, P=0.084; $d.f._{season}$=1, F=3.68, P=0.062; $d.f._{lab\ vs\ field}$=1, F=9.39, P=0.003, Fig. 3). Similarly, tucos in captivity showed higher relative lymphocyte counts than in the field, females had higher lymphocyte counts than males and animals in the reproductive season had lower counts than those in the non-reproductive season (two-way RM-ANOVA: $d.f._{sex}$=1, F=4.70, P=0.035; $d.f._{season}$=1, F=6.77, P=0.013; $d.f._{lab\ vs\ field}$=1, F=13.99, P<0.001, Fig. 3). In the non-reproductive season, stress levels (estimated by N:L ratios) were not significantly correlated with the intensity of the immune response against SRBC neither at day 7 nor at day 14 (day 7: Pearson R=–0.279, d.f.=9, t=–0.822, P=0.435; day 14: Pearson R=–0.198, d.f.=9, t=–0.572, P=0.582). On the contrary, in the reproductive season there was a significant positive association between the intensity of the immune response and N:L ratios at day 7 (Pearson R=0.687, d.f.=12, t=3.138, P=0.009). At day 14, although the association remained positive, it was not significant (Pearson R=0.202, d.f.=12, t=0.685, P=0.507).

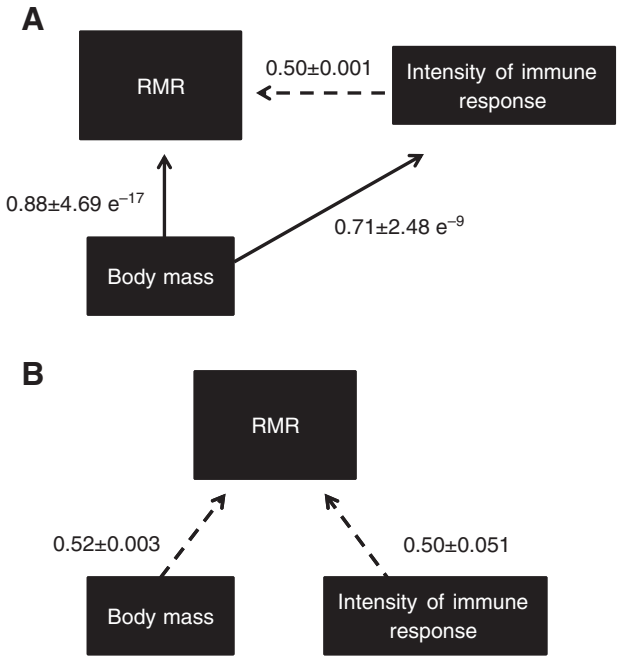

Fig. 2. Best-adjusted path diagrams for resting metabolic rate (RMR), intensity of the immune response against sheep red blood cells (SRBC) (measured as $-\log_2$ antibody titre) and body mass for tuco-tucos injected with SRBC in the non-reproductive (A: 'indirect model') and the reproductive (B: 'direct' model) seasons. Path coefficients (β ± s.e.) are given for each causal relationship. Full lines denote significant paths. Broken lines denote non-significant paths. Variation due to error (ε) is not included for simplicity.

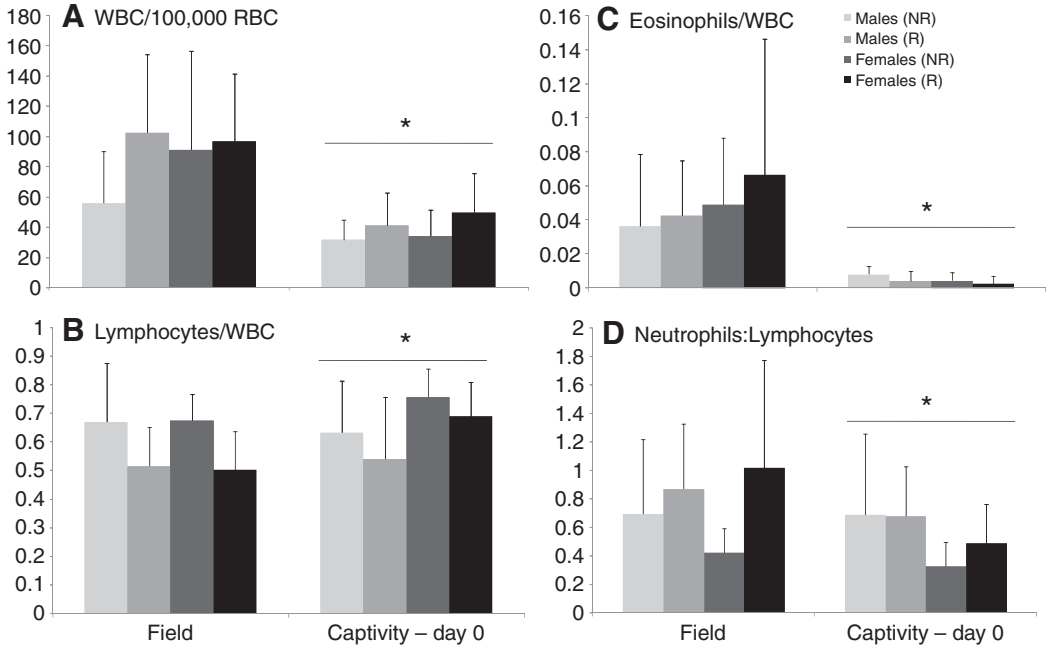

Fig. 3. Means (+ s.d.) of (A) total white blood cell counts (WBC) standardised to 100,000 red blood cells (RBC); (B) relative counts of lymphocyotes; (C) relative counts of eosinophils; and (D) neutrophils:lymphocyte (N:L) ratio in male and female tuco-tucos during the non-reproductive (NR) and the reproductive seasons (R), from blood smears collected in the field, 15 min after capture and after 10 days in captivity. Asterisks denote significant differences between values from the field and from captivity (RM-ANOVA, $P<0.05$).

Haematocrit

Haematocrit did not differ significantly between IC and C animals (three-way RM-ANOVA: d.f.=1, $F=0.08$, $P=0.784$) between males and females (three-way RM-ANOVA: d.f.=1, $F=3.73$, $P=0.06$) or between days of treatment (three-way RM-ANOVA: d.f.=1, $F=0.24$, $P=0.786$) but did differ between the reproductive and the non-reproductive seasons (three-way RM-ANOVA: d.f.=1, $F=17.47$, $P<0.001$).

DISCUSSION

Documenting the extent to which mounting and maintaining an immune response is energetically costly is essential to understand the role of immunological variation and how it relates to life history. The majority of the studies that have assessed the physiological, and particularly the energetic, consequences of an immune challenge in vertebrates are focused on birds (i.e. Ots et al., 2001; Eraud et al., 2005) and relatively less so on mammals – with a bias towards surface-dwelling rodents. Controversial results were obtained from these efforts that led some researchers to propose that the costs of maintaining and even stimulating the immune system are trivial (Williams et al., 1999; Rigby et al., 2002) while others regard the processes involved in immunity as energetically demanding (Lochmiller and Deerenberg, 2000; Bonneaud et al., 2003; Derting and Virk, 2005) and, therefore, plausible to be subject to trade-off decisions (Svensson et al., 1998; Speakman, 2008). Our results are in line with the latter group of studies. The subterranean rodents *C. talarum* (tuco-tucos) are capable of mounting an adaptive immune response against a novel antigen and this response represents a significant energetic cost.

Impact of the immune challenge on energy consumption

Tuco-tucos injected with the non-pathogenic antigen SRBC, which triggers a humoral immune response (Bacon, 1992), mounted a low but detectable primary antibody response 7 d.p.i. The second peak of antibody response, higher than the first, was detected 14 d.p.i. and combined a secondary immune response as well as the primary immune response triggered after the booster injection.

In the non-reproductive season, this antibody response was associated with a significant increase in RMR that ranged from 20 to 35% compared with the RMR of animals before being injected with the antigen. The significant increase in RMR observed in immune-challenged animals is comparable with the cost of lactation for female tuco-tucos [*ca*. 30% (Zenuto et al., 2002a)], which is considered to be the most energetically demanding process in most mammal species (Thompson, 1992). Nonetheless, tuco-tucos that mounted such an expensive immune response against SRBC, in comparison with control animals, did not experience a decrease in their body mass or haematocrit levels, which are predictors of condition (Saino et al., 2000). However, an important fact has to be taken into consideration; tuco-tucos used in this study were maintained in captivity for the duration of the experiments, where they were fed *ad libitum* and kept in plastic boxes where digging was not possible, and they were maintained at controlled ambient temperatures within thermoneutrality. In fact, results from path analysis suggest that body mass, an estimate of an animal condition, may have a significantly positive effect on the intensity of immune response. Those animals that had greater body mass mounted a higher immune response, which was concomitantly associated with a higher increase in RMR. Therefore, one of the questions that remain unanswered is how this significant increase in energy demands associated with an immune challenge would impact the energetic budget of a wild tuco-tuco in natural conditions. In their natural habitat, tuco-tucos have to face other energy demands associated with, for example, thermoregulation, burrow construction and maintenance, and also movements below and above ground. All of these activities, added up to the costs of cellular maintenance, represent a daily energetic expenditure (DEE) of approximately 117 KJ (Table 3). Hence, the exposure to a novel antigen, and the consequent triggering of a specific immune response similar to the one experimentally induced in this study, would represent a 15% increase in the DEE of a tuco-tuco (Table 3). This substantial increase in energy demand in the natural environment may potentially cause a decrease in body condition or an increase in stress levels in wild tuco-tucos if they

Table 3. Daily energetic expenditure in kilojoules (KJ) for male and female *Ctenomys talarum* during a cold day in the breeding and the non-breeding seasons, faced with an immune challenge in their first year of life (modified from Antinuchi et al., 2007)

	Non-breeding female	Pregnant female	Non-breeding male	Breeding male
Maintenance	57.07	57.07	57.07	57.07
Thermoregulation	45.57	45.57	45.57	45.57
Movements	8.73	8.73	8.73	8.73
Burrow construction (1 m)	5.66	5.66	5.66	5.66
Pregnancy	–	13.63	–	–
Immune response at day 10	16.77	10.84	17.90	16.37
Total (KJ day^{-1})	134.00	141.70	134.93	133.40

Only pregnant females were considered in the breeding season, because lactating females were not used in this study. Data on energy costs associated with different processes were obtained from previous studies [cellular maintenance (Luna et al., 2002), thermoregulation (Busch 1989), movements (Luna and Antinuchi, 2003), burrow construction (Luna et al., 2002; Antinuchi et al., 2007), and pregnancy (Zenuto et al., 2002a)].

are not capable of completely covering this demand with their average daily energy assimilation of approximately 160 KJ, obtained from their natural diet (Antinuchi et al., 2007). Interestingly, in this study we found a significantly positive association between the intensity of the immune response and individual stress levels only in the reproductive season, which probably represents the most energetically demanding period for *C. talarum*. Field studies of the impact of an immune challenge in tuco-tucos in natural conditions are then needed to further our understanding of the energetic costs of infections in these animals.

Simultaneous energetic demands: immune challenge during the reproductive season

According to life-history theory, individuals are expected to maximise their fitness by adjusting their investment in current reproduction to a level where the sum of the fitness contributions from future and current reproduction is maximised (Stearns, 1992). In consequence, particularly energetically costly reproductive stages can depress levels of immunocompetence (Degen, 2006; French et al., 2009). Conversely, an induced immune challenge has been shown to negatively impact reproductive behaviour (Bonneaud et al., 2003; Weil et al., 2006). This most likely reflects a trade-off between energy costs of the immune defence and other costly processes involved in reproduction (i.e. Deerenberg et al., 1997; Boonstra et al., 2001; Ardia, 2005; Speakman, 2008).

During the course of this study, we analysed the energetic impact of an immune challenge in both breeding and non-breeding animals. During the reproductive season, only pregnant females were brought from the field to the lab; lactating females caught in the traps were released in their burrows immediately after capture to avoid distressing the pups left in the nest. As mentioned, pregnancy, although costly, does not represent the most expensive reproductive phase for female tuco-tucos, with the greatest daily energetic cost incurred during lactation (Zenuto et al., 2002a). Despite this, the increase in RMR 10 days after the exposure to a novel antigen was lower for female tuco-tucos monitored in the reproductive season (11.55±18.06%) compared with females in the non-reproductive season (35.97±11.20%), as predicted at the beginning of this study. On the contrary, males did not show differences in the levels of increase of RMR associated with the immune challenge in the reproductive season compared with the non-reproductive season.

Focusing on this first set of results only, a lower increase of RMR observed in pregnant females could be interpreted as the outcome of a trade-off in energy allocation between reproductive effort and immunity. Considering that female tuco-tucos give birth to an average of four pups each time and only two times a year at the most (Zenuto et al., 2002a), it may be expected that the balance between the energy demands of reproduction and immunity is biased towards maximising the current reproductive contribution in this species (Stearns, 1992), at the expense of immunocompetence. In future work, we plan to examine the impact of an immune challenge on the energetic expenditure of tuco-tucos subject to ambient temperatures below and above thermoneutrality or to food restriction, to further our understanding of the trade-offs between allocating resources to immunity *vs* other costly processes.

In our work, not only did we monitor the energetic cost of an immune challenge but we also explored the magnitude of the associated antibody response. This led us to observe that, contrary to what is expected under an immunosuppression hypothesis, the lower energetic investment in antibody defence observed in female tuco-tucos during the reproductive season did not appear to affect the intensity of the response against SRBC, estimated by the mean antibody titres against this antigen detected in hemoagglutination assays. One possibility is that pregnancy hormones may have improved the antibody response against SRBC in females at a lower energetic cost compared with the non-reproductive season. Progesterone and prolactin have been observed to stimulate the production of Th2 cells, which are involved in the response triggered by SRBC, to prevent foetal rejection (Roberts et al., 1996; McMurray, 2001). This facilitator effect of pregnancy hormones on Th2-cell production and also humoral immunity could explain how similar antibody titres were obtained from breeding and non-breeding females, despite the fact that the energetic investment on immune response was lower in the first group. Based on these results, we plan to assess the effect of sex hormones on the ability of tuco-tucos to mount an adaptive immune response against a novel antigen in the future.

Implications for interspecific immunological variation

Interspecific variation on the reliance on different components of the immune system has been associated with life-history traits and the ‘speed’ of life (Lee, 2006). ‘Slow-living’ species – with low reproductive rates, high investment per offspring, long developmental times and longer adult lifespans (see Klasing, 2004) – are expected to rely more strongly on induced defenses that require substantial time and resources during development and are mostly beneficial against repeated infections, such as responses mediated by B- and T-cells (Lee, 2006). Tuco-tucos could be considered a ‘slow-living’ species in comparison with some surface-dwelling rodents such as mice and rats; they have long lifespans (Malizia and Busch, 1991), long gestation times [(*ca*. 3 months (Zenuto et al., 2002a)] and give birth to small litters twice a year at the most (Malizia and Busch, 1997; Zenuto et al., 2002b). However, antibody

Table 4. Comparative values of mean antibody titres against sheep red blood cells (SRBC) at the response peak, after 5–15 days post-injection (d.p.i.) for eight species of rodents and two species of birds in the reproductive and the non-reproductive seasons

Species	Mean antibody titres against SRBCs	d.p.i.	Reference
Rodents			
Clethrionomys glareolus (bank voles)	5*, 8†	12	Saino et al., 2000
Mus musculus (mice)	4, 6	5, 15	Bekierkunst et al., 1971
	8	6	Cichon et al., 2002
Peromyscus leucopus (white-footed mice)	8.43	6	Derting and Virk, 2005
Phodopus sungorus sungorus (Siberian hamsters)	7	7	Hadley et al., 2002
Rattus rattus (rats)	7, 9, 10	5, 10, 15	Axelrad, 1968
Ctenomys talarum (Talas tuco-tucos)	1.2, 4.4*	7, 14	Present study
	1.4, 4.6†	7, 14	
Spermophilus parryii plesius (Arctic ground squirrels)	6.8	7	Boonstra et al., 2001;
Cavia porcellus (guinea pigs)	2	7	Nomoto et al., 1980;
Birds			
Parus major (great tits)	1.6±1.55	6–10	Ots et al., 2001
Streptopelia decaocto (collared doves)	6.3±2.58	7	Eraud et al., 2005

*Reproductive.
†Non-reproductive.

titres against SRBC in tuco-tucos seem to be near the lowest end of the spectrum known to date (Table 4).

Tuco-tucos kept in captivity during this study showed a decrease in the numbers of white blood cells and a marked reduction in the numbers of 'rare' leukocytes (e.g. eosinophils). These parameters may reflect an increase in stress levels associated with captivity (see also Vera et al., 2008) and a concomitant immunosuppression (but see Davis et al., 2008). Hence, the ability of tuco-tucos to mount an immune response against SRBC could have been underestimated in this study. However, this does not solely explain the lower levels of antibody titres of tucos-tucos compared with those of other rodents, because most of the studies cited here for comparative purposes were performed in captivity (Table 4). In addition, in this study captive tuco-tucos experienced an increase in their lymphocyte levels compared with those in the field, and the N:L ratio decreased significantly compared with levels measured in animals in their natural habitat, further suggesting that the stress levels associated with captivity, relatively low in our study, do not seem to be the only explanation for the low antibody titres observed in tuco-tucos in comparison with other rodent species.

Differences in life history (Lee, 2006; Martin et al., 2006a) are one of the proposed factors implicated in interspecific immunological variation. Differences in BMR have also been proposed to shape immunological variation within and between species based on the relative costs and benefits of the different immune branches. Lee proposed that large-bodied species with lower mass-specific BMRs should be able to invest more in developmentally costly induced immune responses (Lee, 2006). The energetic costs associated with a humoral response against SRBC have only been examined for one other mammal besides tuco-tucos [white-footed mice (Derting and Virk, 2005)], showing that mounting such a response is also costly for this species. Therefore, extensive work is needed to continue exploring the energetic costs associated with an immune challenge in other rodent species with comparable body sizes and different BMRs to test Lee's prediction (Lee, 2006).

As a final consideration, frequency of parasite challenge should also affect the selective advantage of a certain immune strategy (Shudo and Iwasa, 2001), leading to stronger antibody-mediated responses in species frequently exposed to extracellular pathogens (Janeway et al., 2004). The subterranean environment has been associated with low parasite richness, particularly gastrointestinal helminths (Nevo, 1999). Interestingly, tuco-tucos mounted the lowest antibody response against SRBC compared with other responses reported in the literature (Table 4), suggesting a possible role of the strength of parasite exposure in the evolution of immune defense strategies. However, considering the great increase in RMR associated with mounting an adaptive immune response in tuco-tucos, animals in this study could have mounted a lower antibody response to an artificial stimulation of their immune system with SRBC, which is non-pathogenic (Bacon, 1992) compared with a response to a natural pathogenic infection. Therefore, it is necessary to continue exploring the mechanisms of pathogen and parasite resistance in subterranean rodents to assess the possible association between levels of pathogen exposure and interspecific immunological variation in natural populations of mammals. Specifically, ongoing studies with other antigens, such as well-characterised proteins (e.g. PHA), will contribute to evaluate the costs of a cell-mediated response, which is considered indicative of other diseases, such as intracellular infections [e.g. viruses (Lochmiller et al., 1993)].

This is the first study to assess the impact of an immune challenge on the energy expenditure of a subterranean rodent. Our results showed that mounting an immune response entails significant energetic costs for tuco-tucos, possibly leading to trade-off decisions during energetically demanding periods. However, more work is needed to assess how the immune system of *C. talarum* fights pathogenic antigens and antigens that trigger different immune responses, such as inflammation, to establish how tuco-tucos fit in the general framework of the evolution and maintenance of different immune defence strategies in mammals.

LIST OF ABBREVIATIONS

AIC	Akaike Information Criterion
BMR	basal metabolic rate
C	control
DEE	daily energy expenditure
d.p.i.	days post injection
IC	immune challenged
N:L	neutrophyl to lymphocyte ratio
PBS	phospate buffered saline
RM-ANOVA	repeated-measures analysis of variance
RMR	resting metabolic rate
SRBC	sheep red blood cells
$\dot{V}_{O_2}$	oxygen consumption rate

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